

Metabolic Activation of Benzo(a)pyrene in SENCAR and BALB/c Mouse Embryo Cell Cultures

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The metabolism and DNA binding of benzo(a)pyrene [B(a)P] were compared in early passage mouse embryo cell cultures prepared from SENCAR mice, a strain especially susceptible to two-stage tumorigenesis, and BALB/c mice, a strain relatively resistant to two-stage tumorigenesis. Cultures from both strains metabolized similar amounts of B(a)P; however, the proportion of water-soluble metabolites formed was higher in the BALB/c cultures than in the SENCAR cultures. The major metabolites formed in cultures from both strains were B(a)P-9,10-diol, B(a)P-7,8-diol and the glucuronic acid conjugate of 3-hydroxy-B(a)P. The level of binding of B(a)P to DNA was greater in the SENCAR mouse embryo cell cultures than in the BALB/c cultures after 5, 24, and 48 hr exposure. The major B(a)P-DNA adduct formed in B(a)P-treated cultures from both strains was the adduct formed by reaction of (+)anti-B(a)P-7,8-diol-9,10-epoxide [anti-B(a)PDE, the isomer with the epoxide and the benzylic hydroxyl on opposite faces of the molecule] with the exocyclic amino group of deoxyguanosine. Immobilized boronate chromatography followed by high-performance liquid chromatography demonstrated the presence of small amounts of *syn*-B(a)PDE (the isomer with the epoxide and benzylic hydroxyl on the same face of the molecule)-DNA adducts. The proportions of these amounts were similar in cultures from both strains. The results suggest that SENCAR mouse embryo cell cultures may convert less B(a)P to water-soluble metabolites and more to DNA-binding metabolites than BALB/c mouse embryo cells. If similar differences exist in B(a)P metabolism in the skin of these strains of mice, it could help to explain the relatively high sensitivity of the SENCAR mice to tumor induction by the two-stage protocol.

Introduction

The susceptibility of rodents to tumor induction by carcinogenic chemicals depends on both the species and strain of rodent as well as on the particular type of carcinogen and the route of administration. The two-stage initiation-promotion protocol in mouse skin has been used for over 40 years as an assay for detecting tumor initiators and promoters (1). The speed and sensitivity of this assay were greatly increased by Boutwell and co-workers by breeding strains selected for susceptibility to initiation by 7,12-dimethylbenz(a)-anthracene (DMBA) and promotion by croton oil or its most active component, 12-O-tetradecanoylphorbol-13-acetate (TPA) (1,2). The SENCAR mice developed by this procedure are much more susceptible to tumor induction by this protocol than other mice such as BALB/c mice (3). Slaga et al. (4) have shown that a number of strains differ in their response to this treatment protocol

and that SENCAR mice are much more susceptible than BALB/c mice to both complete and two-stage epidermal carcinogenesis. One component of this difference may be susceptibility to promotion (5), but there is also evidence for strain-dependent differences in the activation of the initiator. Nebert et al. (6) found that the induction of hydrocarbon metabolizing enzymes as well as tumor susceptibility varied in DBA/2 and C57B1 mice. The strain-dependent differences in these enzymes were recently reviewed by Pelkonen and Nebert (7).

Polycyclic aromatic hydrocarbons (PAHs) such as the environmental pollutant benzo(a)pyrene [B(a)P] require metabolic activation to induce biological effects (8,9). To trap the metabolites formed from B(a)P, most studies were carried out either with microsomal fractions from various tissues or in cells in culture (8). Early passage rodent embryo cell cultures were found to have high B(a)P-metabolizing activity (10), and mouse embryo cell cultures were used to study the metabolism and DNA binding of B(a)P and other hydrocarbons (11,12). The first successful method for chromatographic isolation of B(a)P-DNA adducts was developed using DNA from B(a)P-treated mouse embryo cell cultures (13). Recently, techniques have been developed to culture

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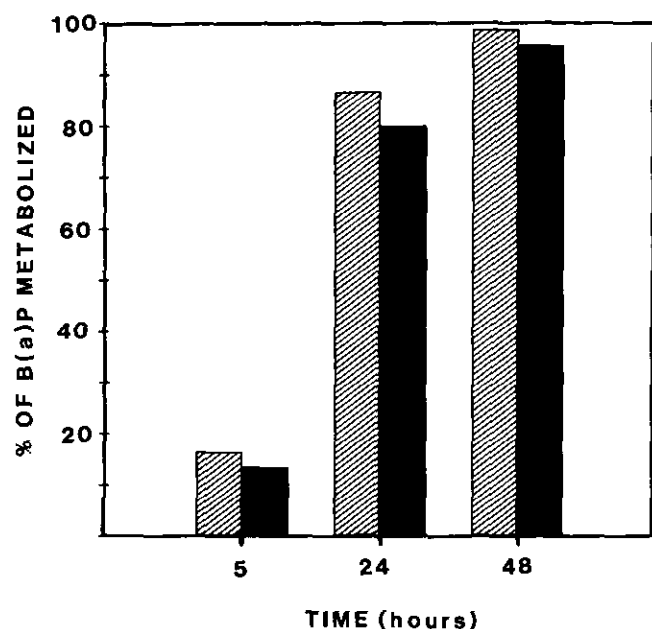


FIGURE 1. Percentage of B(a)P metabolized by BALB/c and SENCAR mouse embryo cell cultures: (▨) BALB/c cell cultures; (■) SENCAR cell cultures. Cultures were exposed to 0.5 μ g [3 H]B(a)P for the times stated, medium samples were removed, extracted with chloroform:methanol and the percentage of B(a)P remaining determined by HPLC.

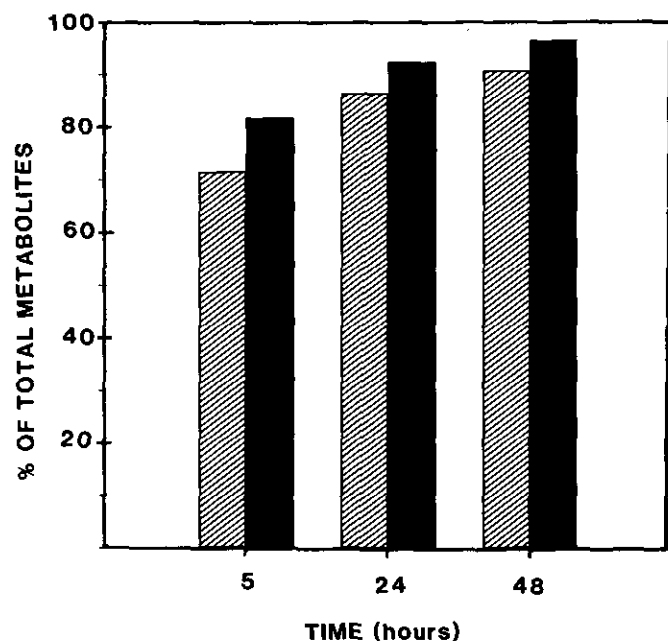


FIGURE 2. Percentage of water-soluble B(a)P metabolites formed in BALB/c and SENCAR mouse embryo cell cultures: (▨) SENCAR cell cultures; (■) BALB/c cell cultures. Cultures were treated with [3 H]B(a)P and analyzed as described in the legend of Figure 1. The percentage of the total B(a)P metabolites recovered in the aqueous phase was calculated.

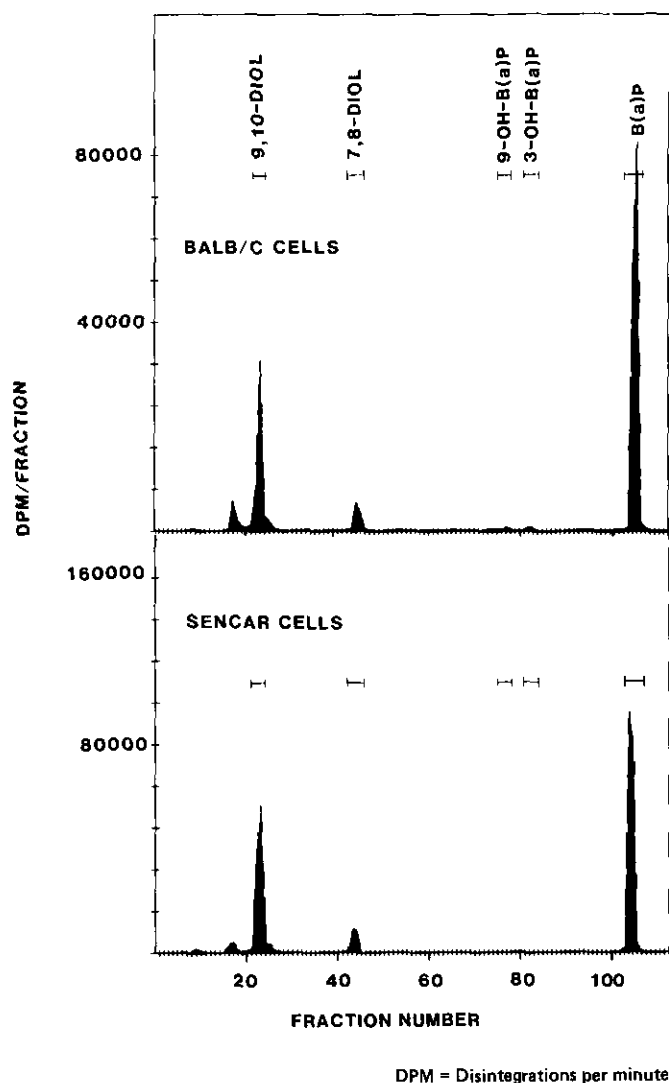


FIGURE 3. HPLC elution profile of the chloroform-extractable B(a)P metabolites formed in BALB/c (top) and SENCAR (bottom) mouse embryo cell cultures. Cultures were exposed to 0.5 μ g [3 H]B(a)P/mL medium for 24 hr and extracted and chromatographed as described in "Materials and Methods." The elution positions of B(a)P metabolite markers are shown by the horizontal bars.

mouse epidermal keratinocytes and to investigate the metabolism and DNA binding of B(a)P in those cells (14-17). Although studies of cells from a number of strains of mice have been performed, B(a)P metabolism and B(a)P-DNA adduct formation in cells from various strains under identical conditions have not been compared directly to assess the role of these factors in the susceptibility of various strains to tumor induction. The present study compared the metabolism of B(a)P in early passage embryo cell cultures prepared from SENCAR mice, a sensitive strain of mice in the two-stage carcinogenesis assay, and BALB/c mice, a relatively resistant strain of mice in this assay (3). The activation of B(a)P to DNA-binding metabolites was also inves-

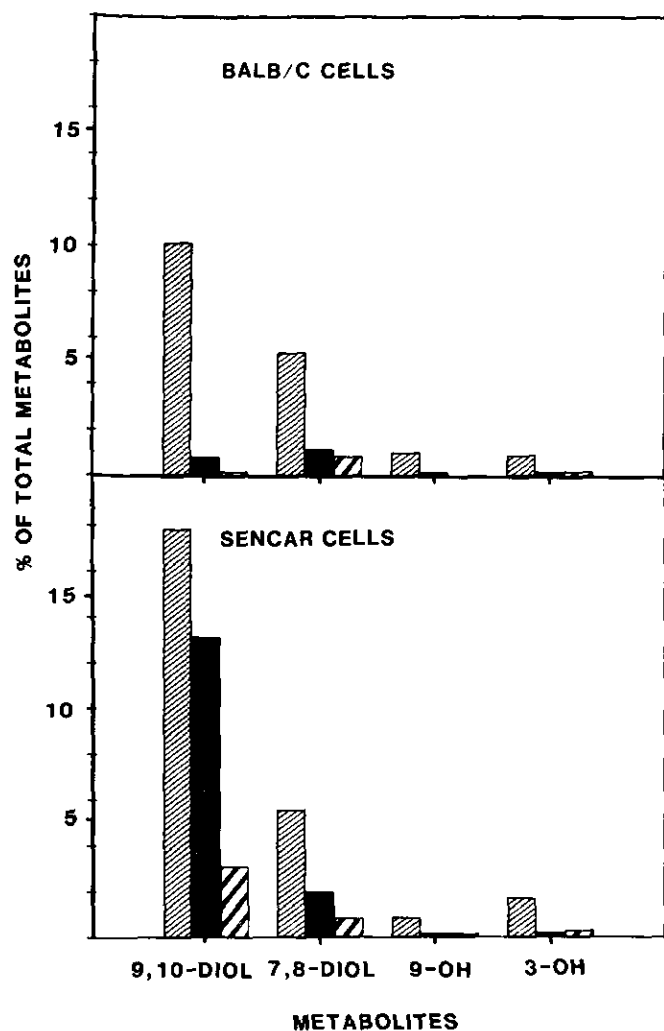


FIGURE 4. Percentage of individual B(a)P metabolites formed in BALB/c (top) and SENCAR (bottom) mouse embryo cell cultures. The metabolites were analyzed as described in the legend of Figure 3. Exposure times were: (▨) 5 hr; (■) 24 hr; (▧) 48 hr.

tigated in cells from these strains using immobilized-boronate chromatography followed by high-performance liquid chromatography (HPLC) (18), a procedure that allows increased resolution of the B(a)P-DNA adducts present.

Materials and Methods

Treatment of Cells

Primary embryo cell cultures were prepared from 15-day SENCAR or BALB/c mouse embryos (Harlan Sprague-Dawley, Inc., Indianapolis, IN) as described previously (18). Tertiary monolayer cultures were prepared in 175-cm² culture flasks with 50 mL minimum Eagle's medium (Grand Island Biological Co., Grand Island, NY) containing 10% fetal bovine serum (Reheis Chemical Co., Phoenix, AZ). [$G-^3H$]B(a)P (Amersham, Arlington Heights, IL) was diluted with B(a)P (Gold

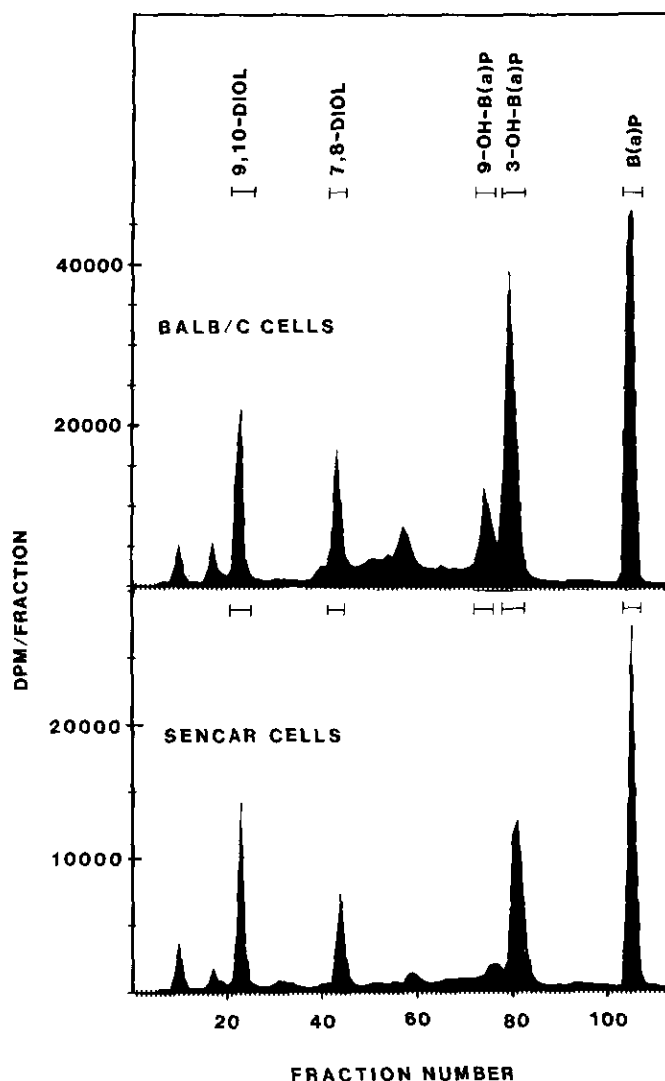


FIGURE 5. HPLC elution profile of the chloroform-extractable B(a)P metabolites present in β -glucuronidase treated medium from BALB/c (top) and SENCAR (bottom) mouse embryo cell cultures. The medium samples were treated with β -glucuronidase as described in "Materials and Methods" before extraction and analysis as described in the legend to Fig. 3. The elution positions of B(a)P metabolite markers are shown by the horizontal bars.

Label; Aldrich Chemical Co., Milwaukee, WI) to a specific activity of 5 Ci/mmol and added to the cultures at a final concentration of 0.5 μ g/mL medium.

B(a)P Metabolism

After exposure of cells for 5, 24, and 48 hr, medium samples were removed, extracted by a two-step chloroform:methanol:water procedure (19), and the amount of radioactivity in aliquots of each phase was determined by liquid scintillation counting. To measure the formation of hydrocarbon glucuronides, medium samples were incubated with 2000 Fishman units of bovine liver β -glucuronidase before extraction (19,20). The chloroform phases were analyzed by HPLC on a Beckman

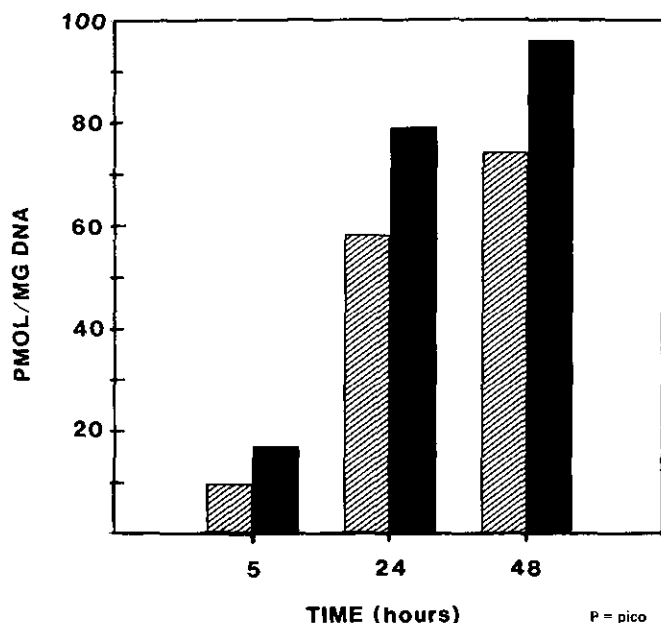


FIGURE 6. Total binding levels of B(a)P to DNA in BALB/c and SENCAR mouse embryo cell cultures: (▨) BALB/c cell cultures; (■) SENCAR cell cultures. Cultures were exposed to [3 H]B(a)P for the times stated, and the DNA was isolated and level of binding determined as described in "Materials and Methods".

Model 312 chromatograph equipped with a 4.6 mm \times 25 cm Ultrasphere octadecyl silane (5 μ m) reverse-phase column (Beckman Instruments, Inc., St. Louis, MO). Samples were eluted at 1 mL/min for 40 min with a linear methanol:water gradient from 11:9 to 17:3 followed by 10 min at 17:3 (20); 110 fractions (0.5 mL) were collected, and radioactivity was determined by liquid scintillation counting. Reference standards of B(a)P metabolites were purchased from the Chemical Repository, Carcinogenesis Research Program, National Cancer Institute.

Analysis of B(a)P-DNA Adducts

After 5, 24, and 48 hr of exposure to B(a)P, the cells were harvested with trypsin:versene, and the nuclei were isolated by homogenization of the pellet in a hypotonic buffer containing Triton-X and stored at -80°C (18). The DNA was isolated by treatment with RNase A (Sigma Chemical Co., St. Louis, MO) and proteinase K (Sigma) followed by CHCl_3 :isoamyl alcohol (24:1, v/v) extraction and ethanol precipitation. After three repetitions, the DNA was precipitated first with 2-ethoxyethanol and then with ethanol (18). The DNA was enzymatically degraded to deoxyribonucleosides (13). B(a)P-deoxyribonucleoside adducts were isolated by chromatography on a Quick-Sep Sephadex LH-20 column (Isolab Inc., Akron, OH) (21). One portion was analyzed by HPLC, and the remainder was analyzed by boronate chromatography before analysis by HPLC.

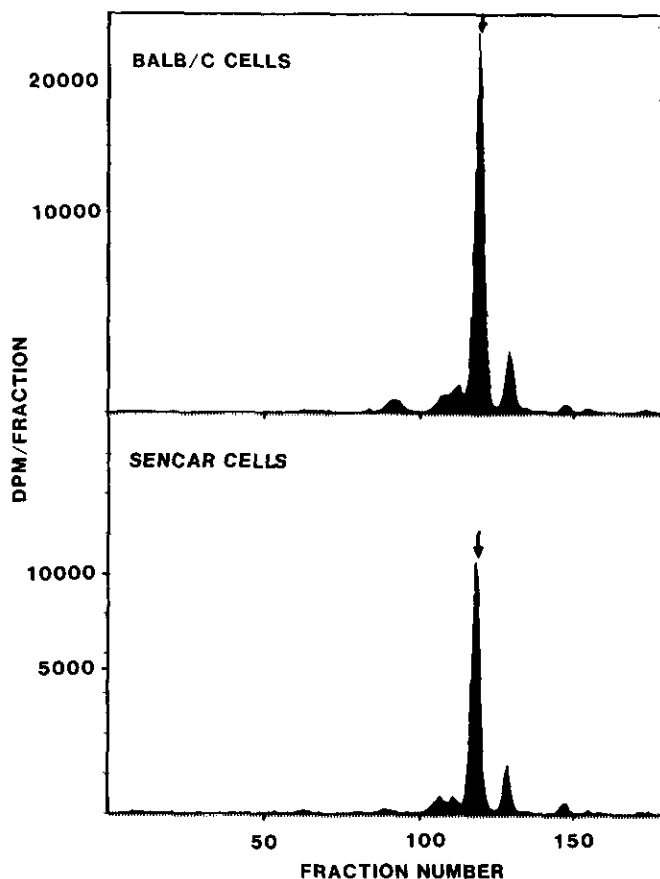


FIGURE 7. HPLC elution profile of B(a)P-DNA adducts formed in BALB/c (top) and SENCAR (bottom) mouse embryo cell cultures. Cultures were exposed to [3 H]B(a)P for 24 hr, and the DNA was isolated, enzymatically degraded to deoxyribonucleosides, and the B(a)P-deoxyribonucleoside adducts analyzed by HPLC as described in "Materials and Methods." The elution position of a [^{14}C](+)*anti*-B(a)PDE-dG adduct that was added to each sample as a marker is shown by the arrow.

HPLC analyses were carried out on a 25 cm \times 4.6 mm Ultrasphere octyl reverse-phase column (Beckman Instruments, Inc., St. Louis, MO). The column was eluted with methanol:water (46:54) for 34 min at a flow rate of 1.0 mL/min, then for 10 min with a linear gradient of methanol:water (46:54 to 55:45), followed by 24 min with methanol:water (55:45) (18). Fifteen 1.0-mL fractions followed by 145 fractions of 0.3 mL each were collected in scintillation vials. Radioactivity was determined by liquid scintillation counting using ACS (Amersham, Arlington Heights, IL). An aliquot of [^{14}C](+)*anti*-B(a)PDE (the isomer of B(a)P-7,8-diol-9,10-epoxide with the epoxide and benzylic hydroxyl on opposite faces of the molecule)-deoxyguanosine (dG) adduct, prepared by reaction of [^{14}C]*anti*-B(a)PDE (National Cancer Institute, Radiochemical Repository) with calf thymus DNA as described previously (21), was added to each sample as a marker.

B(a)P-deoxyribonucleoside adduct samples for boronate chromatography were chromatographed on a 1.0 g column of ([N-(N'-[m-(dihydroxyboryl)phenyl] succi-

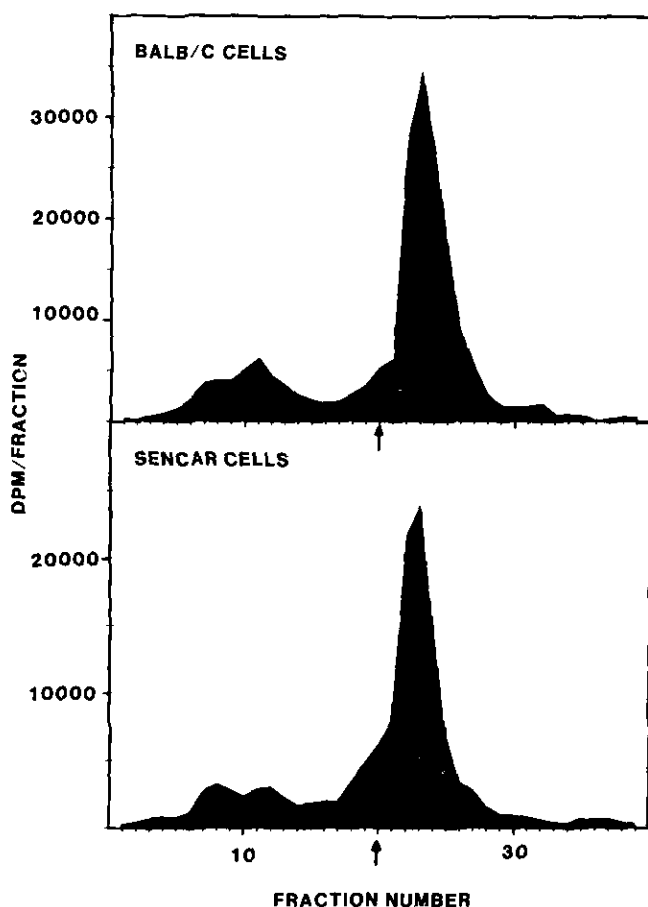


FIGURE 8. Immobilized boronate chromatography elution profile of B(a)P-DNA adducts from BALB/c (top) and SENCAR (bottom) mouse embryo cell cultures. Cultures were exposed to [^3H]B(a)P for 24 hr, and the B(a)P-deoxyribonucleoside adducts were isolated and chromatographed as described in "Materials and Methods." The arrow indicates the fraction number at which sorbitol was added to the eluting buffer.

namyl)amino]ethyl cellulose as described previously (18). The column was eluted with 40 mL of 1 M morpholine (pH 9.0), followed by 40 mL of 1 M morpholine: 10% sorbitol, pH 9.0. Eighty 1.0 mL fractions were collected, and 0.1 mL aliquots of all fractions were removed and placed in scintillation vials. Radioactivity was determined by liquid scintillation counting in Ready-Solv HP (Beckman Instruments, Inc., Fullerton, CA). Buffer fractions containing radioactivity were pooled, applied to a Quik-Sep Sephadex LH-20 column, and the B(a)P-modified deoxyribonucleosides were eluted with methanol (18).

Results

The metabolism of benzo(a)pyrene was compared in confluent third passage cultures of SENCAR and BALB/c mouse embryo cells. The cultures were exposed to [^3H]B(a)P and aliquots of media were removed at 5, 24, and 48 hr for measurement of metabolism. The samples were extracted with chloroform:methanol and

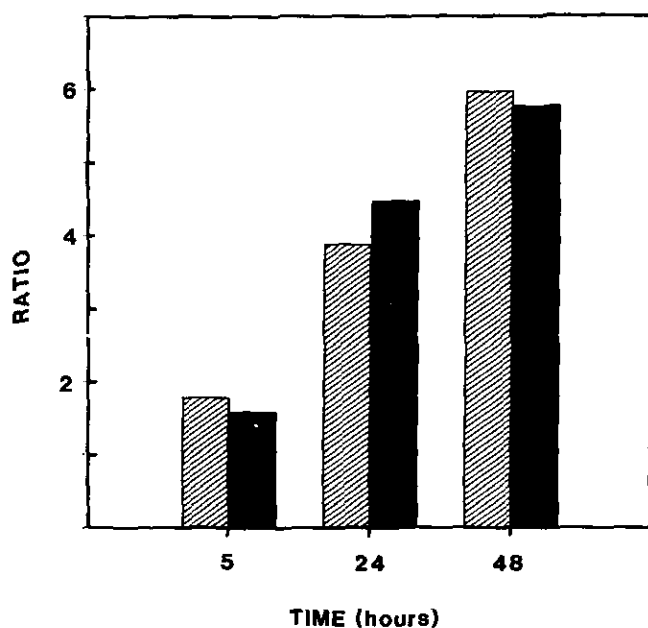


FIGURE 9. Ratio of *anti* to *syn*-B(a)PDE-DNA adducts present in BALB/c and SENCAR mouse embryo cell cultures: (▨) BALB/c cell cultures; (■) SENCAR cell cultures. The cultures were treated and B(a)P-DNA adducts analyzed by immobilized boronate chromatography as described in the legend to Figure 8. The ratio of material eluted in the sorbitol buffer [*anti*-B(a)PDE-DNA adducts] to that eluted in the morpholine buffer [*syn*-B(a)PDE-DNA adducts] was calculated.

the chloroform phase analyzed by HPLC to determine the amount of B(a)P metabolized. The percentages of the B(a)P metabolized are shown in Figure 1. The BALB/c cells appeared to metabolize slightly more B(a)P at all three time points, but none of the differences were statistically significant. Most of the B(a)P was metabolized by cultures from both strains of mice by 24 hr.

The nature of the metabolites formed was investigated by determining the proportion of the metabolites that remained in the aqueous-methanol phase after chloroform extraction. The percentage of water-soluble metabolites at each time point is shown in Figure 2. At all times, the proportion of water-soluble metabolites was higher in the BALB/c than in the SENCAR cells; the increased level in the BALB/c samples was statistically significant in both the 5- and 48-hr samples. The chloroform-extractable metabolites were analyzed by HPLC, and the metabolite profiles are shown in Figure 3. The major metabolites present in cultures from both strains were the 9,10-diol and the 7,8-diol, with only traces of phenolic metabolites present. The percentages of the total metabolites that these two diols and two phenols represent are shown in Figure 4. At the early times, 5 and 24 hr, the SENCAR cell medium contained a higher proportion of 9,10-diol than the BALB/c medium. In both cell types, the highest proportion of these primary metabolites was observed at 5 hr, then this percentage dropped to a very low level by 24 and 48

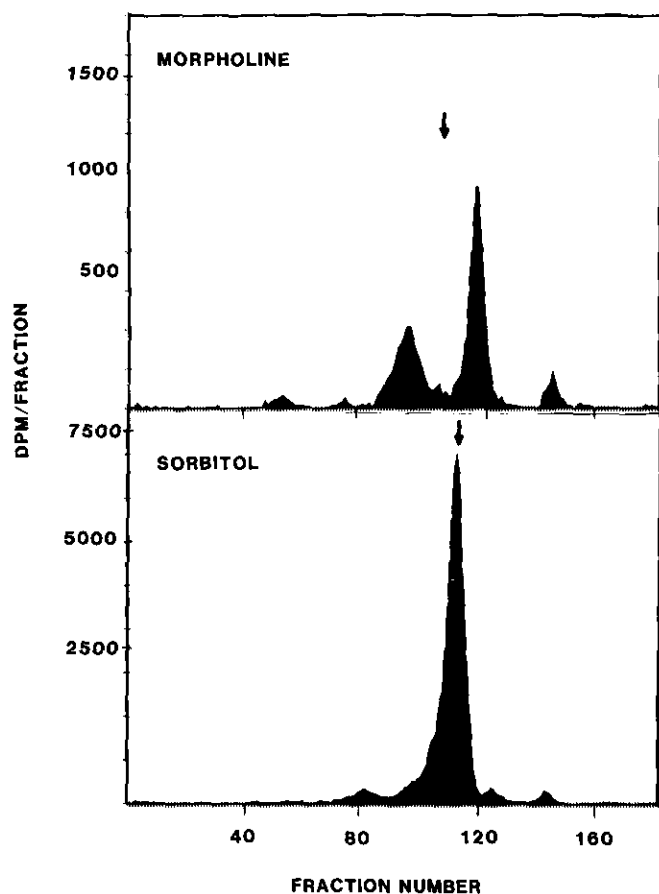


FIGURE 10. HPLC elution profile of the B(a)P-DNA adducts present in the morpholine (top) and sorbitol (bottom) fractions of the immobilized boronate column eluate of (Fig. 8, top) DNA from B(a)P-treated BALB/c mouse embryo cell cultures. The samples were analyzed by HPLC as described in "Materials and Methods." The elution position of a [^{14}C](+)-*anti*-B(a)PDE-dG adduct marker is shown by the arrow.

hr, presumably due to further metabolism of the primary metabolites.

To investigate the nature of the water-soluble metabolites formed in the cultures from these two strains of mice, medium samples were treated with β -glucuronidase to cleave glucuronide conjugates before chloroform extraction. The HPLC profiles of these extracts (Fig. 5) show the presence of three additional peaks, a broad peak in the quinone region (Fractions 50–65) and peaks that chromatographed with markers of 9-hydroxy-B(a)P and 3-hydroxy-B(a)P. The 3-hydroxy-B(a)P peak was much larger than the 9-hydroxy-B(a)P peak in cells from both strains, and the ratio of 9- to 3-hydroxy-B(a)P was similar in both.

The binding of B(a)P to DNA was investigated at 5, 24, and 48 hr. At all times, the SENCAR cells contained a larger amount of B(a)P bound to DNA than the BALB/c cells (Fig. 6). Binding increased from 5 to 24 hr and continued to increase from 24 to 48 hr in spite of the fact that almost 80% of the B(a)P had been metabolized by 24 hr.

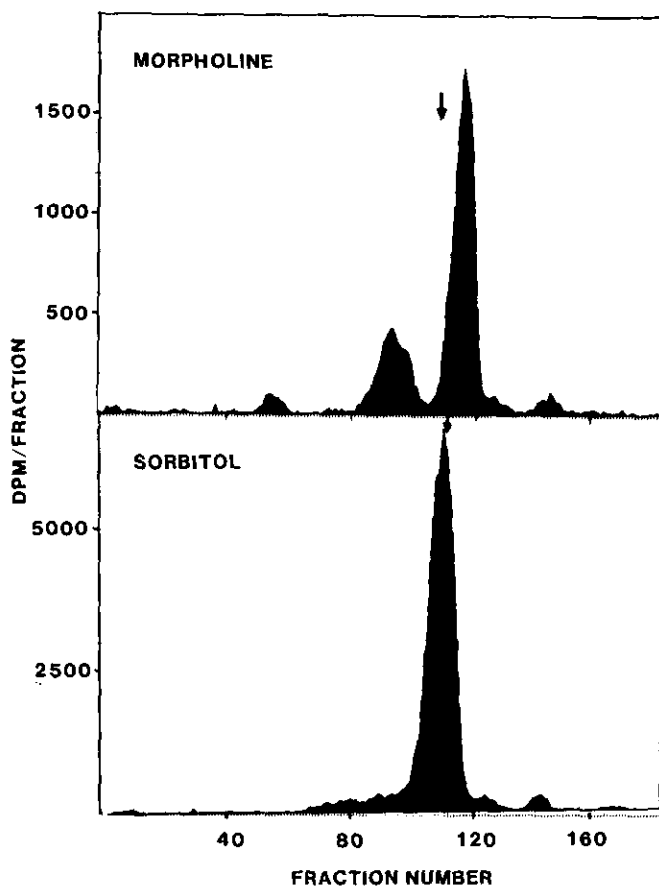


FIGURE 11. HPLC elution profile of the B(a)P-DNA adducts present in the morpholine (top) and sorbitol (bottom) fractions of the immobilized boronate column eluate (Fig. 8, bottom) of DNA from B(a)P-treated SENCAR mouse embryo cell cultures. The samples were analyzed by HPLC as described in "Materials and Methods." The elution position of a [^{14}C](+)-*anti*-B(a)PDE-dG adduct marker is shown by the arrow.

The B(a)P-DNA adducts present were then analyzed by HPLC (Fig. 7). Cells from both strains contained one large adduct peak that coeluted with the adduct formed by reaction of [^{14}C]*anti*-B(a)PDE with the exocyclic amino group of deoxyguanosine (*anti*-B(a)PDE-dG) (9). In addition, several smaller adduct peaks were present, but some were only partially resolved from the *anti*-B(a)PDE-dG peak. To allow the resolution of these minor adduct peaks, the B(a)P-DNA adducts were separated into those which contained *cis* vicinal hydroxyl groups [such as those formed from *anti*-B(a)PDE] and those which did not contain *cis* vicinal hydroxyl groups [such as those formed by *syn*-B(a)PDE, the isomer with the epoxide and benzylic hydroxyl on the same face of the molecule] by immobilized boronate chromatography. The column profiles are shown in Figure 8. Although some material eluted in the morpholine buffer fractions (1–20), the major portion of the adducts eluted after the addition of sorbitol to the buffer (arrow). Thus, the majority of the DNA adducts in both SENCAR and BALB/c mouse embryo cells resulted from binding of the *anti*-isomer of B(a)PDE to DNA. The ratio of sor-

bitol buffer-eluted material to morpholine buffer-eluted material [*anti*- to *syn*-B(a)PDE-DNA adducts] is shown in Figure 9. The ratio increased from less than 2:1 to more than 5:1 in both cell types between 5 and 48 hr, but there was no significant difference between the ratio present in BALB/c cultures and that present in SENCAR cell cultures at any time.

The individual B(a)P-DNA adducts present in the morpholine and sorbitol buffer fractions were analyzed by HPLC on a reverse-phase C_8 column. The profiles for the BALB/c culture DNA samples are shown in Figure 10. The sorbitol buffer fractions contained one major peak that coeluted with the [^{14}C](+)*anti*-B(a)PDE-dG marker and only very small amounts of three other adducts. The one in fraction 140 eluted in the same position as an *anti*-B(a)PDE-deoxyadenosine marker; the others were unidentified. Although the morpholine buffer fractions contained a much smaller proportion of the total material, this material did contain three distinct adduct peaks. The first (fractions 92-98), which was only partially resolved from the *anti*-B(a)PDE-dG peak by HPLC alone (Fig. 7), eluted in the same position as *syn*-B(a)PDE-dG and *syn*-B(a)PDE-deoxycytidine markers. The largest peak (fraction 116-121) eluted in the same position as a marker of *syn*-B(a)PDE-dG, and the small third peak eluted in the same relative position as *syn*-B(a)PDE-deoxyadenosine. The HPLC profiles from immobilized boronate column fractions of the B(a)P-DNA adducts from the SENCAR mouse embryo cell cultures (Fig. 11) contained the same adduct peaks in almost identical proportions. Previous studies have demonstrated that the relative proportions of the three peaks within the morpholine buffer fraction do not change with time. The major time-dependent change in the adducts is the increase in the proportion of the (+)*anti*-B(a)PDE-dG adduct, which is responsible for the time-dependent increase in the ratio of *anti*- to *syn*-B(a)PDE-DNA adducts (Fig. 9).

Discussion

To determine whether strain-specific differences in the metabolic activation of B(a)P could help to explain the difference in tumor susceptibility of BALB/c and SENCAR mice, B(a)P metabolism and DNA binding were investigated in embryo cell cultures prepared from SENCAR and BALB/c mice. The metabolism studies demonstrated that both the SENCAR and BALB/c mouse embryo cell cultures metabolized similar amounts of B(a)P at all three times studied. The only difference in B(a)P metabolism between the cells from the two strains was the higher proportion of water-soluble metabolites formed in the BALB/c cultures. This finding suggests that the BALB/c cells may metabolize the primary diols and phenols more rapidly and thus reduce the proportion activated to DNA-binding derivatives. The organic-solvent-extractable B(a)P metabolites in both cells were similar to those found in SENCAR mouse epidermal cell cultures by DiGiovanni et

al. (15). The relative ratio of 9,10-diol to 7,8-diol was greater in the embryo cell cultures than in the SENCAR epidermal cell cultures (15), but similar to that observed in two epidermal cell lines (15). β -Glucuronidase treatment released mainly 3-hydroxy-B(a)P and a small amount of 9-hydroxy-B(a)P in both SENCAR and BALB/c mouse embryo cell cultures, a result identical to that observed in SENCAR epidermal cell cultures (15). Thus, B(a)P metabolism in the mouse embryo cell cultures was similar to that reported for epidermal cell cultures. The pattern of B(a)P metabolism in mouse embryo cell cultures differed from that observed in Syrian hamster and Wistar rat embryo cell cultures in that cultures from both of the latter species formed a higher proportion of 9-hydroxy-B(a)P glucuronide conjugates (22).

The binding of hydrocarbons to DNA in mouse skin and in mouse embryo cell cultures has been shown to correlate with the carcinogenic potential of the hydrocarbon (11,23). In studies of B(a)P-DNA binding in various strains of mice, however, Phillips et al. (24) found no significant differences in the binding of B(a)P to DNA in strains with varying sensitivity to hydrocarbon carcinogenesis. The level of binding of B(a)P to DNA was greater at all times in the SENCAR mouse embryo cells than in the BALB/c mouse embryo cells, a result that correlates with relative susceptibility of these two strains to tumor induction. The actual B(a)P-DNA adducts formed in both were similar, and the (+)*anti*-B(a)PDE-deoxyguanosine adduct was the major adduct at all times. There were also small amounts of two *syn*-B(a)PDE-deoxyribonucleoside adducts and *syn*- and *anti*-B(a)PDE-deoxyadenosine adducts, but the relative proportions of these were almost identical in cells from both strains. In both the SENCAR and the BALB/c cell cultures there was a time-dependent increase in the proportion of the *anti*-B(a)PDE-DNA adducts, a change that resulted from the increase in the (+)*anti*-B(a)PDE-dG adduct. The profile of adducts formed in the SENCAR mouse embryo cells was very similar to that reported by Ashurst et al. (25) for SENCAR mouse epidermis *in vivo*. The high proportion of the (+)*anti*-B(a)PDE-dG adduct was also consistent with the antibody binding studies of Nakayama et al. (16) in BALB/c epidermis *in vivo*. Thus the binding of B(a)P to DNA in mouse embryo cell cultures appears to be similar to B(a)P-DNA binding in mouse skin.

The B(a)P-DNA adduct profiles in the mouse embryo cell cultures differed dramatically from those formed in Syrian hamster and Wistar and Fisher rat embryo cell cultures (26). Both the rat and hamster cells contained a much more complex profile of adducts and exhibited greater time-dependent alterations in the adducts present. The amount of (+)*anti*-B(a)PDE-dG adduct present in the mouse embryo cell cultures at 24 hr was much greater than that found in either the rat or the hamster embryo cell cultures (26). The (+)enantiomer of *anti*-B(a)PDE is the most potent skin tumor initiator of the B(a)PDE enantiomers (27). The results suggest that both SENCAR and BALB/c mouse embryo cell cultures

form similar proportions of the major DNA adduct of (+)anti-B(a)PDE, but the higher level of binding of B(a)P to DNA in the SENCAR mouse embryo cells may result from formation of larger amounts of this ultimate carcinogen in SENCAR cells than in BALB/c cells. If a larger amount of (+)anti-B(a)PDE is also bound to DNA from SENCAR mouse epidermis, it could help to explain the increased susceptibility of SENCAR mice to two-stage carcinogenesis.

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